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RNA components of *Escherichia coli* degradosome: Evidence for rRNA decay

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ABSTRACT **Recently, we found that a multicomponent ribonucleolytic degradosome complex formed around RNase E, a key mRNA-degrading and 9S RNA-processing enzyme, contains RNA in addition to its protein components. Herein we show that the RNA found in the degradosome consists primarily of rRNA fragments that have a range of distinctive sizes. We further show that rRNA degradation is carried out in the degradosome by RNase E cleavage of A+U-rich single-stranded regions of mature 16S and 23S rRNAs. The 5S rRNA, which is known to be generated by RNase E processing of the 9S precursor, was also identified in the degradosome, but tRNAs, which are not cleaved by RNase E *in vitro*, were absent. Our results, which provide evidence that decay of mature rRNAs occurs in growing *Escherichia coli* cells in the RNA degradosome, implicate RNase E in degradosome-mediated decay.**

RNase E, which is essential for cell growth and was initially characterized as the enzyme that processes 9S RNA to yield a 5S product [p5S RNA (1)], has been shown to control the rate-limiting step in the degradation of a variety of *Escherichia coli* mRNAs (for reviews, see refs. 2 and 3) and a small regulatory RNA, RNAI—an antisense repressor of the replication primer of ColE1 type plasmids (4, 5). Temperature-sensitive mutants have been isolated (6–9) and mapped to its enzymatically active domain (10–12), i.e., the N-terminal half of the polypeptide. Inactivation of RNase E activity in these temperature-sensitive mutants by culture at nonpermissive temperature prolongs the decay of bulk mRNA.

RNase E has been found to be associated in *E. coli* cells with another key RNA-degrading enzyme, polynucleotide phosphorylase (PNPase) (13–16), which has also been shown to interact functionally with RNase E (17). These findings provide support for the earlier notion that RNA degradation in *E. coli* may be mediated by a multicomponent ribonucleolytic complex (18). Recently, a "degradosome" (13, 16, 19) complex that contains not only RNase E and PNPase but also RhlB RNA helicase, enolase (13, 16), DnaK (13, 19), polyphosphate kinase (19), and GroEL (13) has been purified and characterized. In addition to these proteins, RNA was also found to be a component of this degradosome (13).

Herein we report experiments that characterize the RNA associated with the *E. coli* RNase E containing degradosome. Surprisingly, we found that the major part of the RNA components are fragments of stable RNA species, 16S and 23S rRNAs, that along with tRNAs are known to be highly stable under normal growth conditions and are believed to be insensitive to the decay processes that lead to turnover of short half-lived mRNAs (20, 21). We further demonstrate that these stable rRNAs are cleaved *in vitro* by RNase E to yield products similar to those found in the degradosome, strongly suggesting that the degradosome-associated rRNA fragments are RNase E-generated decay intermediates.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The following *E. coli* strains were used: BL21(DE3) [*hsdS gal* (λc*I*ts*857*, *ind1*, *S* am7, *nin5*, *lac*UV5-T7 *gene1*)] and N3433 (22). Plasmid pRE196 (13) contains the FLAG epitope coding sequence fused to the *rne* gene. Transcription of the fusion protein is under control of T7 RNA polymerase promoter as described (13). Plasmid pRE205 is analogous to pRE196 but contains a temperature-sensitive mutation *rne-3071* that causes a Leu → Phe change at position 68 (10, 13). Plasmid pGP1–2 contains the T7 RNA polymerase gene under the control of a temperature-sensitive bacteriophage λ repressor (23). A derivative of the plasmid pT7/T3α-19 (Life Technologies) lacking the intergenic region of bacteriophage f1 was used to clone cDNA. Plasmid pTH90 (24), containing a gene that encodes 9S RNA, was provided by A. von Gabain (University of Vienna).

Expression of the Rne Protein and Purification of the Degradosome and Associated RNAs. FLAG-Rne fusion protein was expressed in *E. coli* BL21(DE3) containing pRE196 or pRE205. Cells were grown in Luria–Bertani broth plus ampicillin (100 μg/ml) to an OD_{600} of 0.6 and then induced with 1 mM isopropyl β-D-thiogalactoside at 30°C for 30 min. Purification of degradosomes was performed as described (13). The degradosome associated RNAs were extracted sequentially by phenol/chloroform and chloroform, mixed with 0.5 vol of 3 M sodium acetate (pH 5.2), and precipitated with ethanol. RNA components were also purified from degradosomes expressed in N3433 strain containing pGP1–2 and pRE196 (or pRE205) as described (13).

Immunoaffinity Purification of RNase E Multicomponent Ribonucleolytic Complex with Anti-FLAG-Rne Antibodies. To purify RNase E multicomponent ribonucleolytic complex from the extract of BL21(DE3) cells lacking the Rne expression vector, anti-FLAG-Rne antibodies were covalently bound (direct coupling) to 0.5 ml of protein A-Sepharose CL-4B (Pharmacia Biotech) and immunoaffinity chromatography was performed with a TBS buffer (50 mM Tris·HCl, pH 7.4/150 mM NaCl)-resuspended mixture of ammonia sulfate-precipitated proteins prepared from growing *E. coli* BL21(DE3) host cells as described (13). An excess of TBS buffer was used for a preelution wash and 0.5 ml of 2% SDS was used to elute bound RNase E complexes. Eluted proteins were analyzed by Western blotting and their associated RNAs

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Abbreviation: PNPase, polynucleotide phosphorylase.
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were purified. The preparation of anti-FLAG-Rne antibodies, immunoaffinity purification of RNase E complexes, and Western blotting were performed as described (25). Anti-PNPase antibodies obtained from George Mackie (University of British Columbia, Vancouver) and anti-RhlB RNA helicase antibodies obtained from Michael Cashel (National Institutes of Health) were used to detect protein components within native degradosomes. Identical procedures but using preimmune serum instead of immune serum anti-FLAG-Rne were performed to detect background RNA binding.

Purification of *E. coli* Ribosomes and rRNAs. *E. coli* ribosomes were prepared from cultures of *E. coli* BL21(DE3) and N3433 as described (26) except that in the second cycle of ammonium sulfate fractionation, the supernatant was collected instead of the pellet. rRNA was extracted sequentially with phenol, phenol/chloroform, and chloroform. After chloroform, extraction was mixed with 0.1 vol of 3 M sodium acetate and then precipitated with ethanol.

Cloning of cDNA, Sequence Analysis, and Southern and Northern Blot Hybridizations. RNA components from degradosomes expressed in BL21 (DE3) strain were used for cDNA cloning. The Super Script Choice system for cDNA synthesis (Life Technologies) was used for randomly primed cDNA synthesis. Sequencing of cDNA and Southern blot hybridization were performed as described (27). Southern blot hybridization was performed sequentially with several probes derived from cDNA clones representing the different regions of 16S and 23S rRNAs.

RNA was separated on 5% denaturing polyacrylamide gels, transferred to Zeta-Probe blotting membranes (Bio-Rad), and hybridized at 50°C for 12 hr with uniformly [^{32}P]UTP-labeled RNA probes containing sequences complementary to 16S or 23S rRNA, which were synthesized *in vitro* from the cDNA corresponding to nucleotides 21–961 of 16S rRNA or nucleotides 1343–2045 of 23S rRNA. The RNA probe used for 5S rRNA detection was complementary to the 9S RNA gene, which was recloned as an *Eco*RI–*Xba*I fragment from plasmid pTH90 (24) into the derivative plasmid of pT7/T3α-19. Hybridization buffer contained 1.5× SSPE (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA), 2.5× Denhardt's solution, 1% SDS, salmon sperm DNA (100 μg/ml), yeast RNA (40 μg/ml), and 50% formamide. Membranes were washed in 0.5× SSC/0.1% SDS at 42°C and subjected to radiography.

Cleavage of RNA by Degradosomes *in Vitro*. Total RNA (6 μg) isolated from *E. coli* BL21(DE3) as described (5) or RNA (6 μg) purified from degradosomes was incubated with 3.4 μg of degradosome protein complex (560 ng/μg of RNA) at 30°C in a final reaction volume of 100 μl containing 20 mM Tris·HCl (pH 7.5), 0.1 mM DTT, 5 mM $MgCl_2$, 100 mM NaCl, 5% glycerol, and 0.1% Triton X-100 (28). *E. coli* tRNA (1 μg) was added to reactions that contained RNA from degradosomes. Reactions were supplemented with 10 mM K_2HPO_4 and 1 mM ATP (16) for PNPase and RhlB helicase, respectively, and incubated at 37°C. Samples (50 μl) were withdrawn 5 min and 15 min after addition of degradosomes. Reactions were stopped by adding 50 μl of phenol/chloroform. RNA was extracted, ethanol-precipitated, dried, and dissolved in 36 μl of formamide-dye loading mixture (95% Formamide, 20 mM EDTA, 0.05% Bromophenol Blue and 0.05% Xylene Cyanol FF), and then 24 μl was electrophoresed on 5% denaturing polyacrylamide gels. In control reactions, 3 μg of RNA was incubated at corresponding conditions for 15 min without addition of degradosomes.

Primer Extension Analysis. Total RNA (1 μg) of BL21 (DE3) was incubated with 560 ng of degradosomes containing wild-type or mutant Rne for 15 min at 30°C in RNase E buffer as described (28). Mutant Rne degradosomes were preincubated for 10 min at 44°C before a 15-min reaction at 44°C. Synthetic oligonucleotides [5′-GGTCTTGCGACGTTATGC-GGT-3′ (complementary to positions 174–194 in the 16S rRNA) and 5′-GCGAGTTCAATTTCACTG-3′ (complementary to positions 2,008–2,025 in the 23S rRNA) were used for both the primer extension and DNA sequencing, as described (29). Clones of cDNA containing 16S (positions 11–679) and 23S (positions 935–1,661) rRNA sequences were used as sequencing templates. A synthetic Su-29-mer (5′-GCTAC-CAGCGGTGGTTTGTTTGCCGGATC-3′) was used for primer-extension analysis for the detection of RNAI and sequencing of pRE196 as described (29).

RESULTS AND DISCUSSION

RNA Components of the Degradosome. FLAG-epitope-tagged Rne (FLAG-Rne) was overexpressed and RNA components associated with FLAG-Rne in degradosomes were purified as described (13). In a control experiment lacking the Rne expression vector, RNA components were not found (data not shown), which is consistent with our previous data (13). RNAs, which consisted of discrete sizes ranging from 100 to 2,800 nucleotides (Fig. 1*a*, lane 1), were analyzed by synthesizing, cloning, and sequencing of corresponding cDNAs. Sequencing analysis of 43 randomly selected clones showed the presence of fragments of mature 16S and 23S rRNA (Fig. 1*c*), and Southern blot analysis of a total of 73 individual cDNA clones showed that 69 clones hybridized with antisense 16S or 23S rRNA [α-^{32}P]RNA probes.

To exclude the possibility that the presence of RNA within the degradosome was caused by an overexpression of FLAG-Rne, immunoaffinity purification of native RNase E multicomponent ribonucleolytic complexes from growing BL21(DE3) cells without RNase E overexpression was performed. Western blot analysis of native RNase E complexes showed the presence of RNase E, PNPase, and RhlB RNA helicase (data not shown). Northern blot analysis of RNA components of native RNase E complexes showed that the RNA components associated with Rne complexes purified

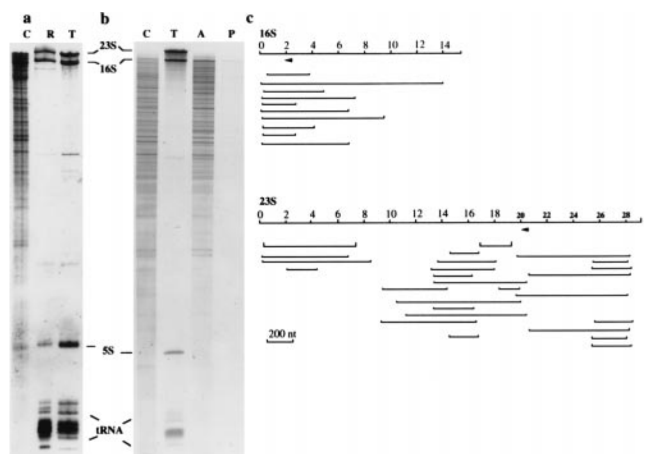

FIG. 1. (*a*) Electrophoretic analysis of RNA associated with FLAG-Rne-containing degradosomes. RNA extracted from degradosomes (lane C), purified ribosomes (lane R), or total lysates of *E. coli* (lane T) was separated on 5% denaturing polyacrylamide gels and stained with ethidium bromide. Positions of 23S, 16S, and 5S rRNAs and tRNAs are shown. (*b*) Electrophoretic analysis of RNA associated with immunoaffinity-purified native Rne complexes. RNA extracted from FLAG-Rne-containing degradosomes and total lysates of *E. coli* (lanes C and T, respectively) from purified native Rne complexes (lane A) were analyzed as described for *a*. Lane P shows a background RNA bound to protein A-Sepharose coupled with the preimmune serum antibodies. (*c*) Clones of cDNA derived from rRNA fragments associated with FLAG-Rne degradosome. Inserts of cDNA clones aligned with previously determined sequences of 16S and 23S rRNA. Arrowheads indicate the positions of oligonucleotide primers.

from extracts of BL21(DE3) cells lacking the Rne expression vector were similar to the RNA components associated with degradosomes containing overexpressed Rne (Fig. 1*b*, lane 3 vs. lane 1).

p5S rRNA and RNAI$_{-5}$, the cleavage products of two well-characterized RNase E substrates, 9S RNA and RNAI, were also detected in degradosome RNAs by Northern blot hybridization (Fig. 2*d*) and RNA primer-extension (Fig. 3) analyses, although their corresponding cDNA was not identified among the characterized cDNA clones. Similarly, the results of primer-extension analysis showed the presence of RNAI decay intermediates consisting of fragments of RNAI molecules (Fig. 3).

In addition to rRNA clones, we identified two clones containing sequences of the *rne* gene (30), one clone corresponding to 10Sa RNA, a small stable RNA encoded by the *ssrA* gene (31–33), and four clones of *malT* gene sequences (34). Identification of the *rne* cDNA indicates that *rne* mRNA is associated with the RNase E complex, in agreement with observations that RNase E cleaves its own mRNA (35, 36). Similarly, the isolation of cDNA clones of fragmented 16S and 23S rRNAs, 10Sa RNA, and *malT* mRNA indicates that these RNAs are associated with degradosomes and suggests that they may be substrates for RNase E.

Northern Blot Analysis of Degradosome RNA Reveals the Presence of Fragments Derived from 23S, 16S, and 5S rRNAs But Not tRNA. The presence of fragments of 16S and 23S rRNAs in degradosomes was confirmed directly by Northern blotting of degradosome RNA; in contrast, only mature 16S and 23S rRNA were detected in purified *E. coli* ribosomes (Fig. 2*a–c*). Similar results were obtained when strain N3433 was used as a source for ribosomes and degradosome RNA (data not shown). A similar hybridization pattern of fragmented 23S and 16S rRNAs was also detected for RNAs isolated from native RNase E (Rne) complexes (Fig. 2*e*). Quantitation by PhosphoImager (Molecular Dynamics) of the overall signals from Northern blot corresponding to RNA extracted from immunoaffinity-purified native Rne complexes (Fig. 2*e*, lane 3) and signals corresponding to background RNA binding (Fig. 2*e*, lane 4) showed that background binding constituted less than 10% of RNA bound to native Rne complexes. The 5S

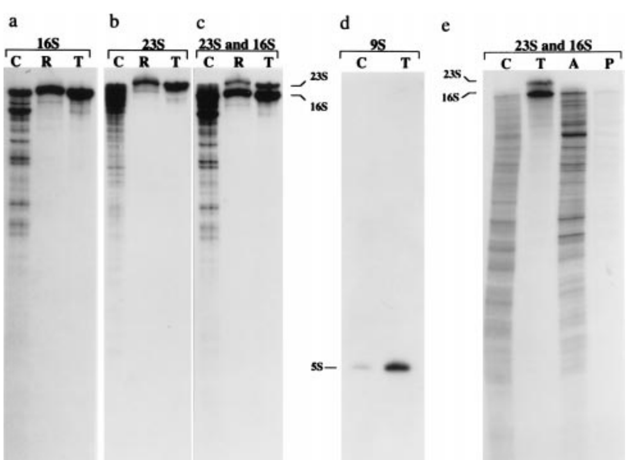

FIG. 2. Northern blot analysis of rRNA associated with degradosomes. RNA extracted from FLAG-Rne-containing degradosomes (lane C), from ribosomes (lane R), or total *E. coli* lysate (lane T) was loaded onto 5% denaturing polyacrylamide gel and resolved by electrophoresis. RNA was transferred to a membrane and probed with uniformly labelled RNAs complementary to 16S (*a*), 23S (*b*), and 16S and 23S (*c* and *e*). (*d*) Results of Northern blot analysis where 9S antisense riboprobe was used. (*e*) Results of Northern blot analysis of RNA associated with immunoaffinity-purified native Rne complexes (lane A; see Fig. 1*b*). Positions of 23S, 16S, and 5S rRNAs are shown.

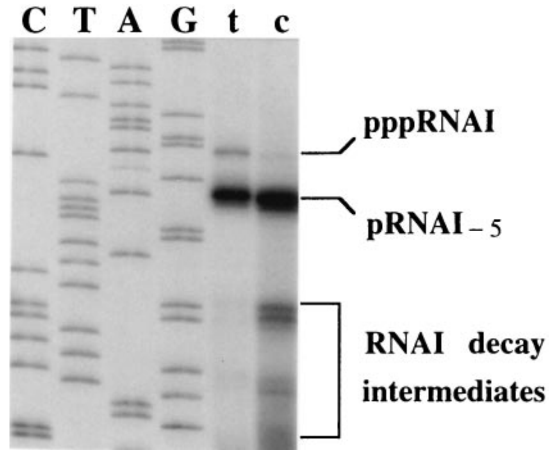

FIG. 3. Detection of RNAI and RNAI$_{-5}$ by primer extension analysis of degradosome RNA components. Degradosome expression was induced in BL21(DE3) strain, containing pRE196. Total RNA (lane t) and RNA components from degradosome (lane c) were analyzed. The positions of pppRNAI, RNAI$_{-5}$, and RNAI decay intermediates are shown.

rRNA was detected both in ribosomes and FLAG-Rne-containing degradosomes (Fig. 2*d*).

Interestingly, the RNA found in degradosomes did not include species the size of tRNAs, which lack cleavage sites for RNase E (Fig. 1 *a*, lane 1, and *b*, lane 3), and Northern blot analysis of degradosome RNA with an α-^{32}P-labeled probe for *supF* tRNA failed to detect tRNA fragments (data not shown). The presence in degradosomes of 5S rRNA, which is known to be a substrate for RNase E *in vivo*, but not tRNAs, which are not substrates, suggested that RNA species cleaved by RNase E may be selectively associated with degradosomes.

Digestion of rRNAs by FLAG-Rne. The notion that 16S and 23S rRNAs are digested by RNase E present in degradosomes was supported by the results of experiments showing that both of these RNAs are cleaved *in vitro* by whole degradosomes in a reaction buffer (28, 37) that allows RNase E activity but precludes digestion by exoribonuclease PNPase, the only other ribonucleolytic enzyme known to be present in degradosomes (Fig. 4*a*, lanes 2 and 3). Cleavage sites on 16S and 23S rRNAs were identified by using oligonucleotide primers complementary to sequences 3′ to single-stranded A+U-rich segments characteristic of regions of RNase E cleavage (2, 40–42) (Fig. 1*c*). As shown in Fig. 4*b*, various RNase E cleavage sites located in A+U-rich single-stranded regions were found in both 16S and 23S rRNAs. Cleavages at the identified sites by whole degradosomes containing temperature-sensitive RNase E (8, 10) did not occur at a nonpermissive temperature (Fig. 4*b*, lanes 5), in which RNase E was inactivated, demonstrating that the observed cleavages were RNase E-dependent. Additionally, many of the sites of RNase E cleavage *in vitro* coincided with the 5′ end of fragmented rRNA species detected in degradosomes (Fig. 4*b*, lanes 3 and 4 vs. lane 6). For example, the cleavage sites mapped in the 5′ end of the 16S rRNA (G11 and U12; Fig. 4 *b*, 16S, lanes 3 and 4, and *c*) were also found by primer-extension analysis of the 5′ end terminus of 16S rRNA components (Fig. 4*b*, 16S, lane 6). Consistently, cDNA clones synthesized from these RNAs lacked the sequence corresponding to the first eight bases of mature 16 rRNA (Fig. 4*b*, 16S, see DNA sequencing ladder).

Digestion of rRNAs by Degradosomes. Along with the cleavage assay in RNase E reaction buffer, we performed a parallel experiment in which degradosome RNA and total RNA were incubated under reaction conditions (16, 28) permissive for the enzymatic activity of RNase E and for the activities of RhlB RNA helicase and PNPase—the other major components of degradosomes (13, 16). We found that after 5

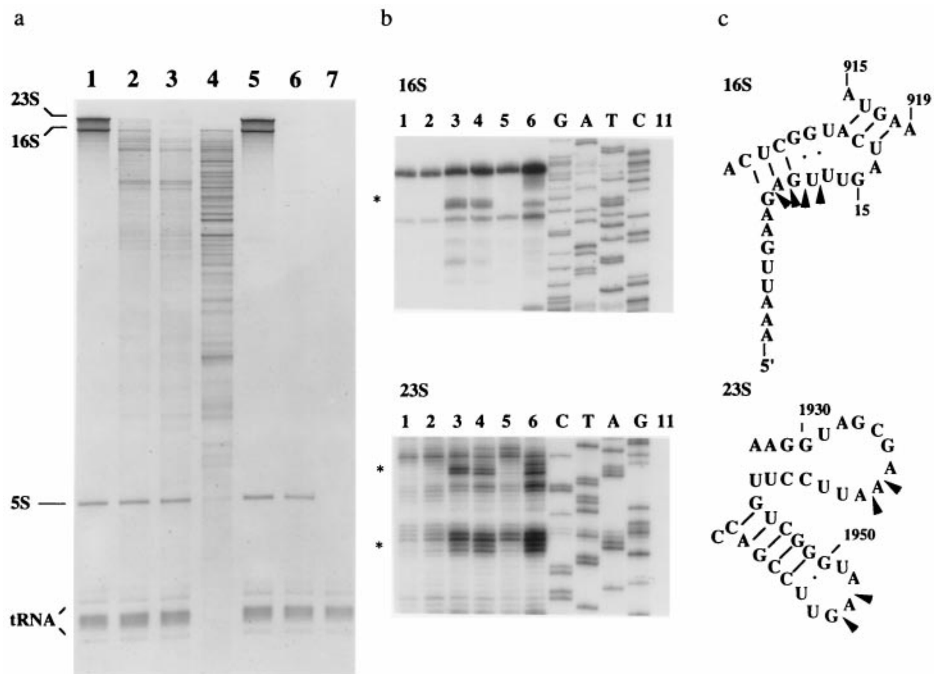

FIG. 4. (*a*) Cleavage of total *E. coli* RNA by FLAG-Rne degradosomes. Total *E. coli* RNA was incubated in RNase E reaction buffer for 15 min at 30°C without (lane 1) or for 5 or 15 min with degradosomes (lanes 2 and 3). Samples for lanes 5–7 were prepared similarly, but reaction buffer was additionally supplemented with ATP and phosphate and incubation was done at 37°C. Lane 4 contains RNA components of degradosome. RNA was separated on gel and stained as described in Fig. 1*a*. Positions of 23S, 16S, and 5S rRNAs are shown. (*b*) Primer-extension analysis of degradosome RNase E cleavages. Total *E. coli* RNA was incubated in RNase E reaction buffer for 0 or 15 min without degradosomes (lanes 1 and 2) at 30°C. Incubations were performed for 15 min at the permissive temperature with degradosomes containing wild-type Rne (lane 3) or mutant (*rne-3071*) Rne (lane 4) or for 15 min at the nonpermissive temperature (44°C) with mutant Rne (lane 5). Lane 6 contains products from a primer-extension reaction of the RNA components isolated from FLAG-Rne degradosomes. Lanes 7–10 contain DNA sequencing ladder. Asterisks indicate fragments produced by RNase E. Aliquots of degradosome were incubated in RNase E buffer for 15 min at 30°C without addition of total RNA (lane 11). (*c*) Segments of folded 16S and 23S rRNAs (refs. 38 and 39) showing the identified cleavage sites.

min of incubation, rRNAs but not tRNAs were degraded (Fig. 4*a*, lanes 5–7) and that 5S rRNA was completely degraded after 15 min (Fig. 4*a*, lane 7). The ability of degradosomes to digest rRNAs but not tRNAs *in vitro* is consistent with our observation that fragmented rRNAs but not tRNAs are found indigenously in degradosomes (Figs. 1 *a* and *b* and 2). Direct evidence for the ability of purified degradosomes to digest native RNA components but not an RNA species (i.e., tRNA) not found in degradosomes is shown in Fig. 5, lanes 2–4. Collectively, our results demonstrate that 16S and 23S rRNAs, which are cleaved by RNase E, and a product generated from 9S RNA precursor by RNase E cleavage (i.e., 5S rRNA) all are degraded in RNA degradosomes in *E. coli* cells.

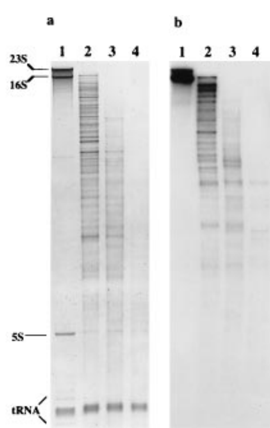

FIG. 5. Cleavage of RNA components by FLAG-Rne containing degradosomes. (*a*) RNA extracted from degradosome was incubated in RNase E reaction buffer supplemented with ATP and phosphate for 15 min at 37°C without (lane 2) and for 5 and 15 min with degradosome (lanes 3 and 4). *E. coli* tRNA was added to reaction mixture as an internal negative control for cleavage (lanes 2–4). Lane 1 contains total *E. coli* RNA. (*b*) Northern blot analysis of cleavage products. RNA probes complementary to 16S and 23S rRNA were used to detect fragmented rRNAs.

Degradation of rRNA is known to occur in *E. coli* cells starved for essential nutrients (21, 43, 44), and 20-year-old observations showing that the rate of synthesis of rRNAs is greater than their rate of accumulation have suggested that these "stable" rRNAs also undergo degradation during active cell growth (45, 46). Recently, rRNA degradation has been shown to occur in *E. coli* mutants lacking PNPase and RNase PH (47). However, the enzyme(s) capable of attacking rRNAs have not been known, and previously there has been no direct evidence for decay of rRNA in normally growing nonmutated bacteria. The experiments reported herein now provide such evidence, show that the multicomponent degradosome complex is involved in this process, and demonstrate that RNase E has a central role in rRNA degradation. Thus, these findings raise important questions about how the previously demonstrated effects of nutrient starvation on rRNA decay are controlled.

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